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(57) Abstract

The present invention discloses methodologies for negative selection of specific phagocytic cells from a heterogeneous population of cells derived from bone marrow and/or peripheral blood utilizing a ferrous–based, preferably carbonyl iron, magnetic selection techniques. Carbonyl iron (CI) selection allows for efficient removal, via the use of a high–energy magnet (e.g., samarium cobalt), of cells such as macrophages, monocytes, and neutrophils without any associated decrease in the number of hematopoietic $CD34^+$ stem or myeloid progenitor cells. CI selection results in the removal of > 40 % of the total number of cells, and a concomitant removal of > 85 % of monocytes in bone marrow samples. Similarly, in peripheral blood samples, CI selection results in the removal of 68 % of the total number of cells, with a concomitant 6–fold enrichment of $CD34^+$. Moreover, when CI selection is combined with Ficoll–Hypaque density gradient centrifugation, a depletion of > 85 % of total cell number and a concomitant 9–fold enrichment of $CD34^+$ cells is found in peripheral blood samples. Following carbonyl iron selection, the enriched cell population may then be subjected to further positive selection of hematopoietic $CD34^+$ stem/progenitor cells and/or negative selection of contaminating, malignant cells utilizing magnetic immunobead methodology.

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MAGNETIC-BASED SELECTION OF HETEROGENEOUS CELL POPULATIONS FOR THE TREATMENT OF MALIGNANT AND HEMATOLOGICAL DISEASES

FIELD OF INVENTION

The present invention relates to methodologies for negative selection of specific cells from a heterogeneous population of cells derived from bone marrow and/or peripheral blood utilizing ferrous particle-based magnetic selection techniques.

BACKGROUND OF THE INVENTION

1. ENRICHMENT OF MONONUCLEAR CELLS FROM A HETEROGENEOUS CELL POPULATION

Negative selection methodologies may be employed to remove various types of phagocytic cells (e.g., macrophages, monocytes, and neutrophils) from lymphoid and non-lymphoid cells in a heterogeneous cell population. These methodologies include: (1) adherence (see Islam, L.N. & Wilkinson, P.C., Evaluation of Methods for Isolating Human Peripheral Blood Monocytes, 121 *J. Immunol. Methods* 75 (1989)); (2) panning using anti-mononuclear monoclonal antibodies (see Ceuppens, J.L. & Baroja, M.L., Monoclonal Antibodies to the CD5 Antigen Provide the Necessary Second Signal for Activation of Isolated Resting T-Cells by Solid-Phase-Bound OKT3, 137 *J. Immunol.* 1816 (1986)) or human gamma globulin (see Chemimi, J., et al., Use of Colloidal Silica (Sepracell-MN) for Enrichment of Dendridic Cells from Human Peripheral Blood: Comparison with Other Methods, 48 *J. Leukocyte Biol.* 74 (1990)), (3) counter-flow centrifugation (see DeMulder,

P.H.M., et al., Monocyte Purification with Counterflow Centrifugation Monitored by Continuous Flow Cytometry, 47 *J. Immunol Methods*, 31 (1981)); (4) phagocytosis of iron particles (carbonyl iron; see Lundgren, G., et al., Differential Effects of Human Granulocytes and Lymphocytes on Human Fibroblasts *In Vitro,* 3 *Clin. Exp. Immunol.* 817 (1968)) or elimination of magnetic particles/beads (Dynabeads M450, Dynal; Oslo, Norway; see Miltenyi, S.. et al., High Purity Magnetic Cell Separation with MACS, 11 *Cytometry* 231 (1990)).

Magnetic-Based Separation Methodologies

Prior to the development of magnetic-based separation methodologies, depletion of specific cells from heterogeneous cell populations derived from bone marrow or peripheral blood has mainly been approached utilizing antibodies conjugated to toxins (e.g., ricin) or with antibodies and associated complement to affect the lysis of "target" cells. See Greaves, M.F., et al., *T and B Lymphocytes: Origins, Properties, and Roles in Immune Responses* (R. Raff, ed) Am. Elsevier, New York, NY pp. 57-63 (1973). These techniques, however, have several disadvantages including the difficulty in quantitatively determining the selective cell destruction in the heterogeneous cell population, non-specific toxicity of either toxins or complement, and the necessity to prepare large quantities of complement In addition, many antibodies are neither cytotoxic with complement nor with various toxin conjugates.

More recently, polymeric microspheres conjugated to antibodies have been utilized to examine cell-surface receptors via electron microscopy. Specifically, iron-containing polymeric microspheres labeled with fluorescent dyes and conjugated to anti-lymphocyte antibodies were used to separate erythrocytes from cells of lymphoid origin by binding of the antibody-microspheres to selected cells and exposing the heterogeneous cell population to a magnetic field. Results demonstrated that ~99% of the antibody-microsphere-bound cells were subsequently attracted to the applied magnetic field, thus effectively sequestering the cells of lymphoid origin. See Molday, M.T., et al., Application of Magnetic Microspheres In Labeling and Separation of Cells, 268 *Nature* 437 (1977). U.S. Patent No. 3,970,518 to Giaever,

discloses the use of magnetic particles conjugated to antibodies to affect the selection of specific viruses, bacteria, or cells from a heterogeneous population of viruses, bacteria, or cells. U.S. Patent No. 4,018,886 to Giaever, discloses the use of small magnetic particles uniformly coated with a protein-specific antibodies which are utilized to provide a large and widely-distributed surface area for the selection of the protein from a solution, thus enabling both the detection and selection thereof when present in low concentrations. Similarly, U.S. Patent 4,230, 685 to Senyel, *et al.*, discloses the use of magnetic microspheres conjugated to protein A.

Additionally, selective depletion of specific cellular constituents from both bone marrow and peripheral blood has been accomplished by the use of magnetic microspheres conjugated to monoclonal antibodies with a flow system using high-energy magnetic selection chambers. This methodology showed initial promise, in that it was far easier to accomplish actual physical removal of the cells rather than to affect selective cell death in a heterogeneous cell population. See Davies, M.J.D. & Parrott, D.M., Preparation and Purification of-Lymphocytes from the Epithelium and Lamina Propria of Human Small Intestine, 22 *Gut* 481 (1981). However, numerous difficulties were encountered in the initial design and utilization of these magnetic separation techniques and their associated system. Difficulties which were encounter included: non-specific binding of the magnetic particles to cells, low overall yields of magnetically-selected cells, inability to remove unbound magnetic particles from the reaction mixture, difficulty in the cleaning and sterilization of the magnetic separation device, and difficultly in removing the antibody-conjugated magnetic particles from the selected cells. See Praganni, F., et al., Large-Scale Collection or Circulating Hematopoietic Progenitor Cells in Cancer Patients Treated with High-Dose Cyclophosphamide and Recombinant Human GM-CSF, 26 *Eur. J. Cancer* 562 (1990); Bregni, M., et al., High Sensitivity and Specificity Assay for the Detection of Human Leukemia and Lymphoma Cells In Bone Marrow, 511 *Ann. New York Acad. Sci.* 473 (1987).

Magnetic-Based Enrichment of a Heterogeneous Cell Population Using Carbonyl Iron

One well-documented magnetic-based cellular enrichment methodology involves the utilization of ferrous (iron) particles or similar preparations of particulate iron or iron crystals which exhibit paramagnetic properties. In this procedure, the heterogeneous cell population is incubated with the ferrous particles which are phagocytized by certain cells (e.g., macrophages. monocytes, or neutrophils). Subsequently, those cells which have phagocytized the ferrous particles may be removed from the heterogeneous cell population with the aid of a powerful magnet. See Levine, S., 123 *Science* 185 (1956). For example, macrophages, being highly phagocytic, are preferentially removed from the heterogeneous cell population due to the fact that lymphocytes do not phagocytize particulate materials. See Greaves, M.F., et al., *T and B Lymphocytes: Origins, Properties, and Roles in Immune Responses* (R. Raff. ed.) Am. Elsevier, New York, NY pp.57-63 (1973).

Previously, carboxyl iron (CI) has been utilized in the aforementioned magnetic-based separation methodologies. Carbonyl iron is a small particulate preparation of highly purified metallic iron which is formed by heating gaseous iron pentacarbonyl ($Fe(CO)_5$), resulting in the deposition of uncharged, sub-microscopic crystals of elemental iron with a chemical formula of; $Fe(CO)_3$ and a particle diameter of $\leq 5\mu m$. See Devasthali, et al., Bioavailability of Carbonyl Iron, 46 *Eur. J. Haematol* 272 (1991). Carbonyl iron has been demonstrated to be phagocytized by various phagocytic cells from the peripheral blood and/or bone marrow, and has been used for depletion of monocytes and macrophages (see Rumpold, H., et al., Influence of Carbonyl Iron Treatment on Lymphocyte Subpopulations, 105 *Archs. Allergy Appl. Immunol.* 105 (1979); Wong, S. & Varesio, R.A., et al., Depletion of Macrophages from Heterogeneous Cell Populations by Use of Carbonyl Iron, 108 *Methods in Enzymol.* 307 (1984)) and for the enrichment of myeloblasts and promyelocytes (see Preisler, H.D. & Epstein, J., A Method for Obtaining Human Bone Marrow Specimens Enriched for Myeloblasts and Promyelocytes, *J. Lab. Clin. Med.* 414 (September, 1979)) or lymphocytes (see Moy, R.T., et al., A Method for Improved Yield and Purity in Extracting Lymphocytes from Lung Tumors, 38 *J.*

Surgical Rev. 17 (1985)). While, the resulting decrease in total cell number following CI selection varies with the concentration of CI utilized, the source of the heterogeneous cell population, and the types of cells present in the heterogeneous cell population, decreases in total cell number typically range from 40-700/9. See Rumpold, H., et al., Influence of Carbonyl Iron Treatment on Lymphocyte Subpopulations, 105 *Archs. Allergy Appl. Immunol.* 105 (1979); Böyum, A., Separation of Leukocytes from Blood and Bone Marrow, 27 *Scand. J. Clin. Lab. Invest.* 1 (1968).

Prior to this invention, however, there were no reports in the literature of the utilization of carbonyl iron to enrich for $CD34^+$ hematopoietic stem/progenitor cells or other myeloid progenitor cells from the bone marrow or peripheral blood for subsequent engraftment into humans. Similarly, nor were there any reports of the use of carbonyl iron to concentrate cellular constituents of bone marrow or peripheral blood stern/progenitor cells (PBSC) for further processing prior to subsequent engraftment in patients. Hence, in conclusion, although carbonyl iron separation of monocytes has been reported in the literature for a period of time, the aforementioned technology has not been utilized for the preparation of the cellular constituents of bone marrow or peripheral blood for further processing or for the concentration of bone marrow or peripheral blood for subsequent clinical use (e.g., hematopoietic rescue via engraftment following high-dose myeloablative chemotherapy).

II. NEOPLASTIC DISEASE

Solid tumors make up approximately 56% of all childhood cancers diagnosed annually in the United States. The main categories are astrocytoma (9.6%), neuroblastoma and ganglioblastoma (7.9%), Wilm's tumor (6.4%), medulloblastoma (4.2%), rhabdomyosarcoma (3.6%), germ cell tumor (3.2%), osteosarcoma (2.6%), and Ewing's sarcoma (2%). See Miller. R.W., et al., Childhood Cancer, 75 *Cancer* 395 (1994). Current treatment methodologies generally include combination chemotherapy and combined modality strategies. For certain cancers, such as Wilm's tumor, a "cure" is achievable in a large majority of patients. Unfortunately, however, most metastatic solid tumors and high-grade malignant brain tumors have

remained resistant to treatment, despite the tremendous advances in the elucidation of the molecular biology of neoplastic disease.

In the last decade, consensus on staging systems has been reached for most pediatric solid tumors. This consensus was accomplished by improved staging methodologies, better definitions of risk groups, and cooperation among treatment centers as in Pediatric Oncology Group (POG) and Children's Cancer Group (CCG) in the United States. See Pediatric Oncology Group: Progress Against Childhood Cancer, 89 *Pediatrics* 597 (1992). Additionally, increasing attention is now being paid to the late effects of treatment. See Bhatia, S., et al., Breast Cancer and Other Secondary Neoplasms After Childhood Hodgkin's Disease, 334 *New Engl. J. Med.* 745 (1996). Moreover, the recent discoveries of unique chromosomal aberrations, cellular oncogenes (e.g., MYC-N in neuroblastoma), tumor suppressor genes (e.g., the CDC2L1 suppressor gene located at chromosomal site 1p36 encoding a 58 kD CDK-related kinase [PITSLRE] in neuroblastoma), as well as autokine growth factors and their receptors (e.g., nerve growth factor [NGF] and brain derived growth factor [BDNF], their receptors TrkA and TrkB, respectively, in neuroblastoma). See Brodeur, G.M., et al., Neuroblastoma, In: *Principles and Practice of Pediatric Oncology, 2nd edition* (P.A. Pizzo & D.G. Poplack, eds.) J.B. Lippincott Co., Philadelphia, PA, pp. 739-749 (1993)). The elucidation of these aforementioned molecular characteristics have served to further the understanding of the highly complex nature of malignant transformation and may potentially provide molecular biological/biochemical "targets" for tumor-selective therapies. See Dai, Y., et al., Cellular and Humoral Responses to Adenoviral Vectors Containing Factor IX Genes, 92 *Proc. Am. Soc. Clin. Oncol.* 1401 (1995). By combining the specificity of immunologic vehicles (antibodies or cells) and the potential for gene manipulation, more efficacious and less toxic treatment modalities may soon be available. See Hwu, P., The Gene Therapy of Cancer, 91 *PPO* (1995); Cheng, N.K.V., Biological and Molecular Approaches to Diagnosis and Treatment of Cancer: Principles of Immunotherapy, In: *Principles and Practice of Pediatric Oncology, 3rd edition* (P.A. Pizzo & D.G. Poplack, eds.) J.B. Lippincott Co., Philadelphia, PA, (1996).

Neuroblastoma

Neuroblastoma arises from neural crest cells that are progenitors of the adrenal medulla and the sympathetic nervous system. It is the most common extracranial (i.e., occurring in the peripheral nervous system (PNS)) solid tumor of childhood and the most common neoplasm diagnosed in the first year of life. Ninety percent of neuroblastoma cases are diagnosed in children 5 years-old or less, with a median age of 2.5 years. See Miller, R.W., et al., A Review of Childhood Cancer, 75 *Cancer* 395 (1994).

Diagnosis of neuroblastoma is typically established by characteristic histopathologic findings, or by tumor cell aggregations in the bone marrow combined with elevation in urinary catecholamies such as vanillylmandelic acid. However, on occasion, more sophisticated techniques are required to rule out other small, round cell tumors such as Ewing's sarcoma (ES), primitive neuroectodermal tumor (PNET), rhabdomyosarconma, and infrequently, lymphoma. See Kushner, B.H. & Cheng, N.K.V., Neuroblastoma: An Overview, 1 *Hematol. Oncol. Annals* 189 (1993).

Unlike many other solid tumors, over 60% of neuroblastomas are already metastatic at the time of initial diagnosis. Massive primary tumors combined with its propensity for metastatic disease give neuroblastoma its dismal clinical reputation because, despite experiencing marked tumor regressions with cytotoxic therapy, most patients eventually die of metastatic disease. Nonetheless, the application of rational treatment modalities, as well as the utilization of novel, targeted therapy (such as that disclosed in this patent application) may change the clinical prognosis of this recalcitrant disease.

SUMMARY OF THE INVENTION

The present invention discloses methodologies for negative selection of specific cells from a heterogeneous population of cells derived from bone marrow and/or peripheral blood utilizing a ferrous-based, preferably carbonyl iron, magnetic selection technique. Carbonyl iron (CI) selection allows for efficient removal, via the use of a high-energy magnet (e.g., samarium cobalt), of cells such as macrophages

and monocytes without any associated decrease in the number of hematopoietic $CD34^+$ stem or myeloid progenitor cells. CI selection results in the removal of $>40\%$ of the total number of cells, and a concomitant removal of $>85\%$ of monocytes in bone marrow samples. Similarly, in peripheral blood samples, CI selection results in the removal of 68% of the total number of cells, with a concomitant 6-fold enrichment of $CD34^+$ cells. Moreover, when CI selection is combined with Ficoll-Hypaque density gradient centrifugation, a depletion of $>85\%$ of total cell number and a concomitant 9-fold enrichment of $CD34^+$ cells is found in peripheral blood samples.

Following carbonyl iron selection, the enriched cell population may then be subject to further positive selection of hematopoietic $CD34^+$ stem/progenitor cells and/or negative selection of contaminating, malignant neuroblastoma cells utilizing the magnetic immunobead methodology disclosed in U.S. Patent No. 4,710,472 to Saur, et al. & Reynolds, C.P., et al., An Immunomagnetic Flow System for Selective Depletion of Cell Populations from Marrow, 17 *Transplant Proc.* 326 (1985). Enrichment for $CD34^+$ cells from bone marrow and/or peripheral blood samples will greatly facilitate hematopoietic rescue in patient undergoing myeloablative chemotherapy. Similarly, removal of contaminating, malignant tumor cells from bone marrow and/or peripheral blood samples will help ameliorate the possibility of tumor reoccurrence in patients undergoing autologous bone marrow or peripheral blood stem cell (PBSC) engraftment. In addition. the selective removal of phagocytic cells from a bone marrow or peripheral blood sample may result in an increased ability to detect extremely low levels of malignant tumor cells in bone marrow or peripheral blood samples, thus facilitating initial diagnosis or subsequent therapeutic treatment.

Therefore, the present carbonyl iron-based magnetic selection provides a rapid and highly effective methodology for the removal or negative selection of phagocytic cells (e.g., macrophages, monocytes, and neutrophils) from heterogeneous cell populations derived from bone marrow or peripheral blood samples.

DESCRIPTION OF THE FIGURES

The present invention may be better understood and its advantages appreciated by those individuals skilled in the relevant art by referring to the accompanying figures wherein:

Figure 1: Illustrates the effect of carbonyl iron (Post-CI) and a combination of carbonyl iron with a Ficoll-Hypaque density gradient separation (Post-CI-FIC) on the total number of cells recovered as compared to pre-selected cells (Pre-F). Also shown is the depiction of $CD34^+$ cells (monocytes) and $CD15^+$ cells (immature granulocytes), as detected by a flow cytometry methodology.

Figure 2: Illustrates the percent of $CD34^+$ or myeloid progenitor cells detected and the overall degree of enrichment following carbonyl iron (Post-CI) and a combination of carbonyl iron with a Ficoll-Hypaque density gradient separation (Post-CI-FIC) as compared to pre-selected cells (Pre-F).

Figure 3: Illustrates the percent of $CD34^+$ or myeloid progenitor cells detected and the overall degree of enrichment (as measured by CFU-GM assay) following carbonyl iron (Post-CI) and a combination of carbonyl iron with a Ficoll-Hypaque density gradient separation (Post-CI-FIC) as compared to pre-selected cells (Pre-F).

Figure 4: Illustrates the total number of $CD34^+$ or myeloid progenitor cells lost (as measured by CFU-GM assay) following carbonyl iron selection (Post-CI) and a combination of carbonyl iron selection with a Ficoll-Hypaque density gradient separation (Post-CI-FIC) as compared to pre-selected cells (Pre-F).

Figure 5: Illustrates percent recovery, total percent detected, and total-fold enrichment of $CD34^+$ or myeloid progenitor cells in peripheral blood samples.

Figure 6: Comparison of the purging efficiency between with carbonyl iron (CI) fraction and without carbonyl iron fraction in three neuroblastoma cell lines (LA-N-1,

LA-N-5, and KCNR) seeded into bone marrow mononuclear cells at a 1% concentration. One cycle of the immunomagnetic methodology was used for purging of the neuroblastoma cells.

DESCRIPTION OF THE INVENTION

Disclosed within the present patent application, is the heretofore unreported use of a ferrous-based magnetic separation methodology for the removal of cells from a heterogeneous cell population derived from biological samples. In this methodology, a heterogeneous population of cells, such as that derived from a bone marrow or peripheral blood sample, is contacted with ferrous particles, preferably carbonyl iron particles, within a temperature range of 4 to 42°C and preferably with continuous agitation. Various phagocytic cells within the heterogeneous cell population (e.g., macrophages, monocytes, and neutrophils) phagocytize the carbonyl iron particles, thus facilitating their subsequent removal by application of a high-energy magnetic field. Following the removal of the sequestered phagocytic cells, the remaining cell population is enriched for other cell types. In addition, the utilization of this methodology does not affect the concentration of hematopoietic stem or myeloid precursor cells found within bone marrow or peripheral blood samples.

In another embodiment of the present invention. the cells which have been selected may be further enriched for $CD34^+$ hematopoietic stem or myeloid progenitor cells by utilization of iron containing polystyrene particles conjugated to antibodies directed against specific cell-surface antigens (e.g., CD34). The magnetic particle/antibody/cell-complexes may then be sequestered by use of an applied magnetic field for subsequent use in hematopoietic rescue following myeloablative therapy.

In yet another embodiment, the selected cells may be "purged" of contaminating, malignant tumor cells by utilizing of an immunomagnetic-based negative selection methodology. Presently, following myeloablative therapy and prior to autologous transplant, neuroblastoma cells are removed from bone marrow and peripheral blood samples utilizing this technique, thus preventing possible

reoccurrence of the tumor. See U.S. Patent No. 4,710,472 to Saur, et al. and Reynolds, C.P., et al., An Immunomagnetic Flow System for Selective Depletion of Cell Populations from Marrow, 17 *Transplant Proc.* 326 (1985).

I. ENRICHMENT OF CD34$^+$ HEMATOPOIETIC/PROGENITOR CELLS FROM BONE MARROW OR PERIPHERAL BLOOD

Isolation of large numbers of CD34$^+$ cells from either the bone marrow or peripheral blood has a variety of therapeutic applications including: (1) purging of malignant cells from peripheral blood or bone marrow cell transplants (see Berenson, R.J., et al., Transplantation of CD34$^+$ Marrow and/or Peripheral Blood Progenitor Cells (PBPC) into Breast Cancer Patients Following High-Dose Chemotherapy (HDC), 82 *Blood* 678 (1993)); (2) purging T-cells from allogenic bone marrow or peripheral blood cell transplants (see Collins, R.H., CD34$^+$ Selected Cells in Clinical Transplants, 12 *Stem Cells* 577 (1993)); and (3) enrichment of stem/progenitor cells for gene therapy (see Bregni, M., et al., Rational and Methods for Retroviral-Mediated Gene Transfer in Peripheral Blood Hematopoietic Progenitors In: *Hematopoietic Stem Cells: Biology and Therapeutic Applications* (D.J. Levit & S. Mertelsmann, eds.) Marcel Decker, New York, NY pp. 185-194 (1995)) and in the generation of function antigen-presenting dendridic cells (see Siena, S., et al., Massive *Ex Vivo* Generation of Functional Dendridic Cells From Mobilized CD34$^+$ Peripheral Blood Progenitors for Anticancer Therapy, 23 *Exp. Hematol.* 1463 (1995)).

CD34 is a cell-surface sialomucin-like, single chain transmembrane glycophosphoprotein expressed on developmentally early lymphohematopoietic stem and progenitor cells, small-vessel endothelial cells, and embryonic fibroblasts possessing a molecular weight of 105-120 kD, depending upon the degree of glycosylation. CD34 recognizes at least 3 different epitopes and the 40 kD protein core is highly N-and O-glycosylated. See Civin, C.I., et al., A Hematopoietic Progenitor Cell-Surface Antigen Defined by a Monoclonal Antibody Raised Against KG-la Cells, 133 *J. Immunol.* 157 (1984).

Recent CD34 functional studies have demonstrated that CD34 expressed on endothelial cells may play a role in leukocyte adhesion and "homing" during inflammatory processes. See Find, L., et al., Expression of the CD34 Gene in Vascular Endothelial Cells, 75 *Blood* 2417 (1990). It has also been postulated that CD34 functions as a leukocyte cytoadhesion molecule which interacts with CD62E and CD62L, playing a possible role in stem/progenitor cells localization/adhesion in the bone marrow. See Krause, D.S., et al., CD34: Structure, Biology, and Clinical Utility, 87 *J. Amer. Soc. Hematol.* 1 (1996). CD34 may also be involved in maintenance of the hematopoietic stem/progenitor cell phenotype. See Fackler, M., et al., Full-Length, but not Truncated CD34, Inhibits Hematopoietic Cell Differentiation of M1 Cells, 85 *Blood* 3040 (1995). Despite the importance of CD34 as a cell-surface marker of early hematopoietic stem/progenitor cells in experimental and clinical hematopoiesis, the putative biological functions of CD34 have not yet been fully elucidated.

While $CD34^+$ bone marrow cells comprise only 1.5 to 3% of marrow mononuclear cells and ≤ 1 % of peripheral blood mononuclear cells, they provide precursors for all lymphohematopoietic lineages as purified $CD34^+$ cells have been shown to be able to reconstitute all lineages after myeloablative chemotherapy in humans (see Dunbar, E., et al., Retrovirally-Marked CD34-Enriched Peripheral Blood and Bone Marrow Cells Contribute to Long-Term Engraftment After Autologous Transplantation, 85 *Blood* 3048 (1995), Berenson, R.J., et al., Engraftment After Infusion of $CD34^+$ Marrow Cells In Patients with Breast Cancer and Neuroblastoma, 80 *Blood* 1717 (1991)), thus confirming that cells for both transient and long-term hematopoietic repopulation are found in the $CD34^+$ compartment.

Recently, the rapid kinetics of hematologic recovery following myeloablative chemotherapy and the comparative ease of collection using a variety of mobilization schemes have been well-documented advantages of utilizing stem/progenitor cells isolated from the peripheral blood (peripheral blood stem cells, PBSC), rather than bone marrow, to facilitate hematopoietic rescue. Collection of PBSC has been described by Bensinger, W., et al., Autologous Transplantation with Peripheral Blood

Mononuclear Cells Collected After Administration of Recombinant Granulocyte Stimulating Factor, 81 *Blood* 3158 (1993). However, because of the larger volumes of cells involved in PBSC harvests, as PBSC preparations which possess the ability to restore hematopoiesis generally contain between 2 to 10 times the number of cells that an autologous marrow preparation contains in order to provide the number of progenitor cells needed to restore hematopoiesis following myeloablative therapy. See Williams, S.F., et al., Selection and Expansion of Peripheral Blood $CD34^+$ Cells in Autologous Stem Cell Transplantation for Breast Cancer, 87 *Blood* 1687 (1996). Thus, higher numbers of cells must be processed and eventually infused into the patient, unless the initial processing step enriches for $CD34^+$ stem cells.

Typically, median granulocyte aplasias range between approximately 9 and 13 days after re-infusion of enriched PBSC (to reach 500 granulocytes/μL). Even more impressive are the median times reported for platelet engraftment using this methodology, ranging between 10 and 15 days, which is significantly shorter than the time required with bone marrow transplantation. See Beyer, J., et al., Hematopoietic Rescue After High-Dose Chemotherapy using Autologous Peripheral-Blood Progenitor Cells or Bone Marrow, 6 *J. Clin. Oncol.* 1328 (1995). The reasons for the rapid hematologic recovery after PBSC transplantation and the mechanism for the concomitant mobilization of hematopoietic stem cells is unclear at this time. The rapid engraftment could result merely from increased numbers, compared to bone marrow components, of mature progenitor cells which quickly give rise to peripheral blood cells. Conversely, there may be intrinsic functional differences in the stem cells which result from the process of mobilization into the peripheral blood. Accessory cells may also be different both in number and function, although the rapid engraftment observed despite $CD34^+$ cell enrichment would militate against large quantities of accessory cells being the cause of the rapid engraftment. Patients who receive PBSC collected in "steady state" or following chemotherapy mobilization without the addition of hematopoietic cytokines (e.g., G-CSF or GM-CSF) generally experience longer periods of aplasia, with median times for granulocyte and platelet engraftment of 20.5 to 25 days and 25 to 38 days, respectively. See Korbling, M., et al., Autologous Blood Stem Cell Transplantation in Patients with

Advanced Hodgkin's Disease, 8 *J. Clin. Oncol.* 987 (1990). Again, this difference in engraftment kinetics could be a function of the numbers of stem/progenitor cells harvested, or the administration of cytokines during the mobilization may alter the function of stem/progenitor cells found in the periphery to more rapid or more complete adhesion to the bone marrow stroma or faster growth once the cells become reestablished in the marrow compartment. Additionally, minor phenotypic differences have been demonstrated between bone marrow and peripheral blood stem cells by several investigators. These phenotypic differences include decreased expression of *c-kit* on $CD34^+$ cells from the peripheral blood in comparison to those in the bone marrow. See To, L.B., et al., A Comparative Study of the Phenotype and Proliferative Capacity of Peripheral Blood (PB) $CD34^+$ Cells Mobilized by Four Different Protocols and Those of Steady-State PB and Bone Marrow $CD34^+$ Cells, 84 *Blood* 2930 (1994). Exposure to some cytokines has also been shown to alter the stromal adhesion capacity of cell cytoskeleton integrin molecules, thus possibly allowing mobilization of stem/progenitor cells into the peripheral blood and/or more rapid "homing" into the marrow space. See Kovach, N.L., et al., Stem Cell Factor Modulates Avidity of $\alpha 4\beta 1$ and $\alpha 5\beta 1$ Integrins Expressed on Hematopoietic Cell Lines, 85 *Blood* 159 (1995). Nonetheless, not all transplant centers have reported rapid hematologic recovery following $CD34^+$ PBSC engraftment. See Juttner. C.A., et al., Circulating Autologous Stem Cells Collected in Very Early Remission from Acute Non-Lymphoblastic Leukemia Produce Prompt but Incomplete Hematopoietic Reconstitution, 61 *Br. J. Haematol.* 739 (1985). The kinetics of engraftment, however, can be predicted by enumeration of the hematopoietic progenitor cells infused and the most rapid engraftment occurs when the PBSC component contains above a threshold number of hematopoietic cells as determined by quantitiation of $CD34^+$ cells or myeloid progenitor cells cultured *in vitro* (CFU-GM).

Various selection methodologies have been employed to select/enrich for mobilized $CD34^+$ stem/progenitor cells in peripheral blood. Procedures for $CD34^+$ cell enrichment are defined as positive or negative by selection of CD34 cell-surface antigen positive (e.g., expressing) or negative (e.g., non-expressing) cells, respectively. Among negative selection methodologies, investigators have attempted

to concentrate hematopoietic $CD34^+$ stem/progenitor cells by exploiting differences in cell size and density, as well as functional properties between $CD34^+$ cells and other cellular elements. Generally, these negative selection methodologies have employed density gradient centrifugation to separate the mononuclear cell layer (which contains the $CD34^+$ stem/progenitor cells) from granulocytes and erythrocytes utilizing Ficoll-Hypaque (see Pharmacia Fine Chemicals, Booklet - *In Vitro Isolation of Lymphocytes*, (1983)) or Percoll (see Schriber, J.R., et al., Enrichment of Bone Marrow and Blood Progenitor Cells by Density Gradients with Sufficient Yields for Transplantation, 23 *Exp. Hematol.* 1024 (1995)) as the density gradient media. These negative selection methodologies generally provide an unacceptably low yield of $CD34^+$ cells with losses of up to 50% of hematopoietic stem/progenitor cells. Similarly, negative selection methodologies employing adhesion to glass-or nylon-wool fiber exhibits only a $\leq$ 65% recovery of $CD34^+$ cells. However, it should be noted that this latter procedure appears to be more effective in the selection of $CD34^+$ cells than that provided by density gradient centrifugation. See Di Nicola, M. et al, Combined Negative and Positive Selection of Mobilized $CD34^+$ Blood Cells, 94 *Br. J. Haematol.* 716 (1996).

The development of monoclonal antibodies which recognize $CD34^+$ hematopoietic stem/progenitor cells (see Baum, C.M., et al., Isolation of a Candidate Human Hematopoietic Stem Cell Population, 89 *Proc. Nat'l Acad. Sci.* 2804 (1992)), have greatly enhanced procedures which can specifically isolate these antibody-sequestered cells from both the bone marrow and peripheral blood. A number of methodologies have been utilized for the positive selection of cells "labeled" with anti-CD34 monoclonal antibodies. These methodologies involving positive selection include: (1) fluorescence-activated cell sorting (FACS; see Civin, C.R. & Loken, M.R., Cell Surface Antigens on Human Stem Cells: Dissection of Hematopoietic Development Using Monoclonal Antibodies and Multiparameter Flow Cytometry, 5 *International J. Cell Cloning* 267 (1987)) -- which returns a population of highly purified $CD34^+$ cells at the expense of overall speed and total yield, or (2) immunoadsorportion to an avidin-biotin solid phase support (CellPro; see Berenson, R.J., et al., Positive Selection of Viable Cell Population Using

Avidin-Biotin Immunoadsorption, 91 *J Immunol. Methods* 11 (1986)) or micro-magnetic particulate beads (MiniMacs; see Miltenyi, S., et al., High Purity Magnetic Cell Separation with MACS, 11 *Cytometry* 231 (1990)).

II. NEGATIVE SELECTION OF CONTAMINATING MALIGNANT CELLS

For many drug-responsive tumors, chemotherapy can achieve tumor response, but relapse of the tumor frequently occurs. While administering increased dosages of the chemotherapeutic agent(s) can ameliorate larger numbers of tumor cells, such treatment frequently results in greater bone marrow toxicity causing severe myelosupression or even total marrow destruction (myeloablative). See Frei, E., et al., Preclinical Studies and Clinical Correlation of the Effect of Alkylating Dose, 48 *Cancer Res.* 6417 (1988). To shorten the duration of severe myelosupression and restore bone marrow function following high-dose myeloablative chemotherapy, the patient may be infused with their own cryopreserved bone marrow or, more recently, with autologous hematopoietic stem/progenitor cells collected from the marrow or mobilized and collected from the peripheral blood, or combinations of both. Currently, myeloablative therapy consisting of high-dose chemotherapy, followed by hematologic restoration with autologous stem cell or autologous bone marrow transplantation, have become important treatment modalities in many patients with aggressive hematological malignancies (e.g., acute myologenous leukemia, multiple myeloma) and solid tumors (e.g., neuroblastoma. breast cancer). For a review see Ruiz-Argüelles, G.J., et al., Current Treatment Modalities in Neoplastic Disease, In: *Haematology 1996* (J.R. McArthur, ed.) pp. 327-356 Singapore Scientific Press (1997).

However, these aforementioned treatment modalities frequently result in early, post-transplant reoccurrence of malignant disease, most probably resulting from residual tumor cells within the autologous graft. See Brugger, W., et al., Mobilization of Tumor Cells and Hematopoietic Progenitor Cells into Peripheral Blood of Patients with Solid Tumors, 8 *Blood* 636 (1994). Post-transplant tumor

reoccurrence has been shown with numerous neoplastic diseases including acute myologenous leukemia (AML, see Brenner, M.K., et al., Gene-Marking to Trace Origin of Relapse After Autologous Bone Marrow Transplantation, 341 *Lancet* 85 (1993)); chronic myologenous leukemia (CML, see Deisseroth, A.B., et al., Genetic Marking Shows That Ph^+ Cells Present in the Autologous Transplants of Chronic Myologenous Leukemia (CML) Contribute to Relapse After Autologous Bone Marrow Transplant in CML, 83 *Blood* 3068 (1994); follicular lymphoma (see Gribben, J.G., et al., Immunologic Purging of Marrow Assessed by PCR Before Autologous Bone Marrow Transplantation for B-Cell Lymphoma, 325 *New Engl. J. Med.* 1525 (1991); and neuroblastoma (see Rill, D.R., et al., Direct Demonstration that Autologous Bone Marrow Transplantation for Solid Tumors can Return a Multiplicity of Tumorogenic Cells, 84 *Blood* 3068 (1994).

For example, a major difficulty in the autologous engraftment of neuroblastoma patients with bone marrow or PBSC is contamination of the transplanted material with occult tumor cells. Gene-marking experiments have clearly established that tumor-contaminated marrow is at least partially responsible for relapse. See Moss, T.J., et al., Clonogenicity of Circulating Neuroblastoma Cells: Implications Regarding Peripheral Blood Stem Cell Transplantation, 83 *Blood* 3085 (1994); Brenner, M.K., et al., Gene Marking to Determine Whether Autologous Marrow Infusion Restores Long-Term Hematopoiesis in Cancer Patients, 342 *Lancet* 1134 (1993). Therefore, the high degree of peripheralization of clonogenic neuroblasts in neuroblastoma has prompted the utilization of *ex vivo* purging of harvested bone marrow or peripheral blood stem cells (PBSC) of contaminating, malignant tumor cells.

Another approach, as previously discussed, is to utilize positive selection of $CD34^+$ hematopoietic stem/progenitor cells, from either the bone marrow or peripheral blood, which possess the ability to restore marrow function. However, as previously discussed, there has recently been a trend away from use of bone marrow transplantation to support myeloablative therapy, and engraftment with enriched stem/progenitor cells from peripheral blood (PBSC) is now becoming widespread. Generally, PBSC are easier and less painful to collect, result in rapid restoration of

hematopoietic function, and may potentially have less tumor contamination. This latter assumption has prompted a number of investigators to use collected PBSC without the subsequent purging of any contaminating tumor cells. Nonetheless, various studies have demonstrated varying degrees of tumor contamination in PBSC, thus potentially causing a reoccurrence of the tumor. See Bird, J.M., et al., Tumor Contamination in Peripheral Blood and Bone Marrow in Patients Diagnosed with Neoplastic Disease: A Review, 15 *Eur. Mol. Biol. Organi. J.* 316 (1994).

As previously discussed, one potential difficulty in purging or selectively collecting $CD34^+$ stem/progenitor cells from peripheral blood is that the cell numbers obtained from the patient I . n a PBSC preparation are about 2 to 10 times that of a marrow collection, in order to get a sufficient quantity of stem cells to optimally restore marrow hematopoietic function. Therefore, methods to purge tumor cells from PBSC or, alternately, the selective collection of $CD34^+$ cells from peripheral blood, are hampered by the large cell numbers one must manipulate. Moreover, even if purging or $CD34^+$ selection was not used for peripheral blood, the concentration of the cell numbers would greatly facilitate storage of the product and infusion into the patient.

Methods to selectively remove tumor cells from bone marrow or PBSC preparations have only been in clinical use for approximately 10 years. Typical selection methodologies currently include: pharmacological approaches; immunologic methods, usually with concomitant use of complement. to selectively destroy the contaminating tumor cells (see Gribben, J.G., et al., Immunologic Purging of Marrow Assessed by PCR Before Autologous Bone Marrow Transplantation for B-Cell Lymphoma, 25 *New Engl. J. Med.* 1525 (1991)); attachment of antibodies to a solid matrix in columns (see Billadeau, D., et al., Detection and Quantitation of Malignant Cells in the Peripheral Blood of Patients with Multiple Myeloma, 80 *Blood* 1818 (1992)) or most commonly, magnetic "immunobeads" to facilitate the physical removal of the contaminating tumor cells (see Reynolds, C.P., et al., An Immunomagnetic Flow System for Selective Depletion of Cell Populations from Marrow, 17 *Transplant. Proc.* 326 (1985)).

Another example of a highly effective separation methodology to selectively remove tumor cells from bone marrow or peripheral blood has recently been developed. This technology has been disclosed in U.S. Patent 4,710,472 to Saur, et al. and Reynolds, C.P., et al., An Immunomagnetic Flow System for Selective Depletion of Cell Populations from Marrow, 17 *Transplant. Proc.* 326 (1985), both of which are incorporated herein by reference. These references disclose a magnetic based separation methodology to remove specific cells, to which monoclonal antibody-conjugated microspheres have been previously bound, from a heterogeneous cell population. For example, contaminating, malignant neuroblastoma and T-cell leukemia cells have been removed from both bone marrow and peripheral blood using this methodology -- providing a 7 to 10 log and 4 to 4.8 log depletion of the neuroblastoma and T-cell leukemia cells, respectively. In addition, this methodology is currently utilized in the purging of neuroblastoma from bone marrow for the Children's Cancer Group, a cooperative study group of academic institutions that together care for about one-half of children with cancer in the United States. See Peake, I.A., Treatment Of Childhood Cancer, In: *Haematology* 1996 (J.R. McArthur, ed.) pp. 102-111 Singapore Scientific Press (1997).

While it is theoretically possible that collections of hematopoietic progenitor cells from the peripheral blood may contain fewer numbers of malignant cells than bone marrow collections, in some neuroblastoma patients the total number of malignant cells in the peripheral blood may equal or even exceed the number in bone marrow. See Moss, T.J., et al., Contamination of Peripheral Blood Stem Cell Harvests by Circulating Neuroblastoma Cells, 76 *Blood* 1879 (1990). A recent study has demonstrated that, in 46 patients with solid tumors treated with intensive alkylating agent-based chemotherapy, the maximal number of tumor cells found in the peripheral blood occurred at day 5 in patients *with no* tumor involvement in the bone marrow and none of the peripheral blood samples contained tumor cells after day 9, whereas the maximal number occurred at day 10 for those patients *with* marrow involvement. See Bruger, W., et al., Mobilization of Tumor Cells and Hematopoietic Progenitor Cells into Peripheral Blood of Patients with Solid Tumors,

83 *Blood* (1994). Hence, while increased dosages and/or cycles of chemotherapy may decrease the number of malignant cells found in the peripheral blood, there may also be a concomitant decrease in the number of PBSC harvested for subsequent engraftment.

Nonetheless, very little quantitative investigation the purging of contaminating tumor cells from PBSC preparations has been performed, principally because of the larger volumes of cells involved in the harvesting of stem/progenitor cells from peripheral blood. As was previously discussed, PBSC preparations which possess the ability to restore hematopoietic function following myeloablative chemotherapy generally contain between 2 to 10 times the number of cells that an autologous marrow preparation contains, in order to provide the number of progenitor cells needed to restore hematopoiesis. See Williams, S.F., et al., Selection and Expansion of Peripheral Blood CD34+ Cells in Autologous Stem Cell Transplantation for Breast Cancer, 87 *Blood* 1687 (1996). Thus, higher numbers of cells must be processed and eventually infused into the patient. unless the initial processing step enriches for $CD34^+$ cells.

Use of $CD34^+$ selection to concentrate the hematopoietic stem cells has been used successfully in several clinical trials, and sequestering of the $CD34^+$ cells has generally utilized either a solid phase column or use of magnetic beads coated with anti-CD34 antibody. See Rowley, S.D., Overview of Peripheral Blood Stem Cell Collection and Storage, In: *Haematology* 1996 (J.R. McArthur, ed.) pp. 290-294 Singapore Scientific Press (1997).Although patients have been shown to engraft with $CD34^+$ selected cell preparations, it is as yet unclear if restoration of hematopoiesis in such patients is delayed compared to either unseparated marrow or *PBSC* preparations, or marrow or PBSC that is purged of tumor cells, leaving most of the hematopoietic cells in the graft. Moreover, as $CD34^+$ selected preparations have been shown to have tumor cell contamination, even after using $CD34^+$ selection, the purging of tumor cells may still be required to ensure that tumor reoccurrence does not occur.

EXPERIMENTAL METHODOLOGIES

The following section will disclose the various experimental methodologies utilized in the present invention. While then methodologies were primarily applied to bone marrow and peripheral blood samples, they could equally well be applied to the study of other biological samples. Additionally, while malignant neuroblastoma cells were utilized, any other type of malignant cells and/or tissues potentially could be used with equal efficacy.

Example 1: Bone Marrow and Peripheral Blood Stem Cell Isolation

Whole bone marrow cells were obtained from patients who were undergoing bone marrow harvests at Children's Hospital in Los Angeles (CHLA) in which the excess marrow harvested could be utilized for research purposes with prior informed consent. Peripheral blood stem cells (PBSC) were collected by leukapheresis at CHLA using sodium citrate (ACD) as an anticoagulant. Aliquots of ~$2x10^8$ cells were placed in a 15 ml plastic tube and stored at room temperature overnight. The cells were then washed twice with Hank's balanced salt solution (HBSS), resuspended in HBSS, and counted using a trypan blue dye exclusion to determine viability and Turk's solution to count nucleated cells. Viability of $CD34^+$ or myeloid progenitor cells was found to be 99-100% by the trypan blue dye exclusion methodology.

Example 2: Carbonyl Iron Enrichment of Bone Marrow or Peripheral Blood

A total of $1x10^8$ nucleated bone marrow or PBSC cells were placed into a 15 ml polystyrene plastic tube and 200 mg of carbonyl iron (CI; C-3518, Sigma, St. Louis, MO) was added to the tube and the final volume brought to 10 ml with Leibovitz's L-15 medium (Gibco BRL, Life Technologies, Grand Island, NY) pre-warmed to 37°C and supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Life Technologies). Carbonyl iron was used either without sterilization after washing in 95% ethanol or after sterilization by steam autoclaving. Incubation in carbonyl iron was carried out by placing the tube horizontally in the 37°C incubator for 1 hour. Alternately, incubation can be carried out with agitation using a rocker

platform in the 37°C incubator for 1 hour. The carbonyl iron and cells which attach to it are then attached to the sides of the tube using the magnetic field from two opposing high-energy (e.g., samarium cobalt) magnets as described in U.S. Patent No. 4,710,472 for 3-5 minutes. Suspended cells not adhering to the sides of the tube were then transferred to a new tube of the same volume. The original tube was then washed twice by gentle addition of 5 mi each of the same supplemented L-15 medium to recover all CI-free cells. Cells collected after separation were washed twice in supplemented L-15 medium before further treatment.

For larger scale (i.e., clinical scale) magnetic separation of cells using carbonyl iron cells were treated with carbonyl iron (~200 mg CI/10^8 cells) in plastic blood transfer packs, incubated with agitation (either rocker platform, rotary shaker, or rotation), and then subjected to rotational magnetic separation in a blood bag sized magnetic separator, followed by draining the unseparated cells from the bag past a column of opposing high energy (e.g., samarium cobalt) magnets as described in U.S. Patent No. 4,710,472.

For cell separation by Ficoll-Hypaque (Pharmacia AB, Upsula, Sweden) density gradient centrifugation, the cells were layered onto the Ficoll-Hypaque (density 1.077 g/ml) and centrifuged for 20 minutes at 1500 rpm in the room temperature. Following centrifugation, the cells at the Ficoll-Hypaque medium interface were collected and transferred to a tube and washed twice with L-15 medium supplemented with 10% FBS.

After the aforementioned separation steps, the number of hematopoietic stem/progenitor cells were counted and the viability calculated using the trypan blue dye exclusion and nucleated cell numbers determined using Turks solution. Samples of cells from each step during the procedure were set aside for other assays.

Example 3: Determination of $CD34^+$ Stem/Progenitor Cells

Before staining the cells, mature erythrocytes were lysed by incubation in 0.83% ammonium chloride solution for 2 minutes. The remaining cells were then washed twice with L-15 medium supplemented with 10% FCS. Triplicate samples of ~$5x10^5$ cells were stained for each different analysis. Quantitation of the number

of $CD34^+$ stem/progenitor cells was performed by two-color flow cytometry in which mature leukocytes were identified and "gated out" of the analysis. Three phycoerythrin (PE) conjugated mouse anti-human leukocyte monoclonal antibodies (CD3, CD11b and CD14; Becton Dickinson, San Jose, CA) were utilized to exclude mature leukocytic cells. The $CD34^+$ stem/progenitor cell population was then identified using two mouse anti-human fluorescence isothiocyanate (FITC) conjugated CD34 antibodies (HPCA-2, Becton Dickinson, Q Bend-10, GenTrak, Inc.).

Negative controls for PE and FITC were also used. The PE-negative control consisted of 10 μl of PE-mouse anti-human IgG (Becton Dickinson) and 5 μl each of the two FITC-CD34 antibodies used to stain $5x10^5$ cells, whereas the FITC-negative control consisted of 10 μl of FITC-mouse anti-human Ig and 2.5 μl each of the three PE-conjugated lineage positive antibodies (PE-CD3, CD11b and CD14) which were used to stain another aliquot of $5x10^5$ cells.

Cells were pre-incubated with human IgG at a concentration of 1μg IgG/10^6 cells at 4°C for 30 minutes to block the F_c receptors present on the cell membrane prior to their incubation with the aforementioned PE-and FITC-conjugated antibodies. For $CD34^+$ cell determination, 2.5 μl of each of three lineage positive antibodies (PE-CD3, CD11B, CD14) and 5 μl each of the two CD34 antibodies (FITC-conjugated) were used to stain $5x10^5$ cells at room temperature for 15 minutes, protected from light. Cells were then washed twice with Dulbecco's Phosphate Buffered Saline (PBS, pH 7.4; Irvine Scientific, Santa Ana, CA) solution (without calcium and magnesium) supplemented with 5% heat inactivated goat serum (Sigma, St. Louis, MO) and 0.1% of sodium azide and fixed with 2% paraformaldehyde in PBS (adjusted to pH 7.0), and stored for up to 1 week at 4°C in the dark prior to subsequent flow cytometry analysis. A minimum of 10,000 events were collected in list mode for each parameter and the data were saved into the 3.5" floppy diskettes. Using the PC-LYSYS software (Becton Dickinson), gates were set to collect the "lymphocyte" population (low forward scatter), and from that specific cell population only FITC positive cells which were concomitantly negative for PE staining with CD3, CD11B, CD14 were counted as $CD34^+$ cells.

Example 4: Colony-Forming Unit (CFU) Assay

The CFU-GM (Colony Forming Unit-granulocyte and macrophage) assay was used to determine the clonogenic ability of recovered myeloid progenitors. Individual assays were performed in triplicate to help ensure statistical relevance. Cells from either before or after separation (~$2x10^5$ nucleated cells/dish) were plated onto 0.3% of agar containing 400 ng/ml of rhCSF (recombinant, human granulocyte and macrophage Colony Stimulating Factor, Immunex Corporation, Seattle, WA), 17% of heat inactivated FBS, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, 10^{-4} mole/L of α-thioglycerol in a total volume of 1 ml per dish. The cells were then incubated in 37°C incubator at 5% CO_2 and 100% of humidity for 14 days before the individual colonies ($\geq$ 50 cells) were enumerated.

Example 5: Determination of Residual Carbonyl Iron Following Separation

The supernatant cells, recovered after the previously described magnetic separation procedure, were centrifuged at a speed of 2,000 rpm for 10 minutes, and the cell pellets were then resuspended in 100 μL of L-15 medium. The cells were examined by light microscopy with the aid of a samarium cobalt magnet, as particles which were "movable" with the magnet were considered to be remaining carbonyl iron particles.

Example 6: Purging of Neuroblastoma Cells "Seeded" into Bone Marrow

Aspirated bone marrow was seeded with a neuroblastoma cell line pre-labeled with Hoechst 33342 (see U.S. Patent No. 4,710,472) and using the indirect immunomagnetic method reported previously (see Reynolds et al., An Immunomagnetic Flow System for Selective Depletion of Cell Populations from Marrow, 17 *Transplant Proc.* 139 (1986)), the effect of carbonyl iron separation on tumor cell purging efficacy was the evaluated. Statistical analysis was performed by use of the Students t-test to compare the significance between two determined mean values.

EXPERIMENTAL RESULTS

I. Carbonyl Iron-Based Enrichment of Bone Marrow Preparations

Figure 1 shows the effect of carbonyl iron (Post-CI) and a combination of carbonyl iron with a Ficoll-Hypaque density gradient separation (Post-CI-FIC) on the total number of cells recovered. Also shown is the depletion of CD14+ cells (monocytes) and CD15+ cells (immature granulocytes), as detected by a flow cytometry methodology. As may be seen, carbonyl iron removes ≥40% of the total number of cells in the bone marrow and ≥85% of monocytes.

In contrast, as shown in Figures 2 and 3, the concentration of $CD34^+$ or myeloid progenitor cells (as measured by CFU-GM assay) is increased in the CI or in the CI-FIC separation procedures, with CI alone providing a 2-fold enrichment and CI-FIC providing a 3-fold enrichment.

As shown in Figure 4, in spite of the decrease in total cell number, there is no detectable-loss of $CD34^+$ or myeloid progenitor cells when the total number of $CD34^+$ or CFU-GM cells recovered is compared to pre-separated cells.

II Carbonyl Iron-Based Enrichment of PBSC Preparations

The enrichment achieved by CI was demonstrated to be even greater in peripheral blood stem cell (PBSC) preparations. As shown in Figure 5, CI removes approximately 68% of the total number of cells, and achieves a 6-fold enrichment of $CD34^+$ cells. Hence, CI treatment of a PBSC preparation decreases the total number of cells in the preparation to one-third of the initial starting value without a detectable loss of hematopoietic progenitor cells.

III. Effect of Carbonyl Iron Pre-Treatment on the Efficacy of Immunomagnetic Purging of Neuroblastoma Cells

As it was previously demonstrated that CI pre-treatment could significantly decrease the amount of reagents needed for bone marrow purging, the effect of CI pre-treatment on the efficacy of purging was next examined. As shown in Figure 6, CI pre-treatment does not significantly effect the ability of immunobeads to remove

neuroblastoma cells from bone marrow although there is a trend towards the CI-treated preparations showing a greater degree of tumor removal with magnetic immunobeads. This may be due to removal of cells by the CI which would non-specifically bind to immunobeads and decrease their availability to bind to tumor cells in the bone marrow.

While embodiments and applications of the present invention have been described in some detail by way of illustration and example for purposes of clarity and understanding, it would be apparent to those individuals whom are skilled within the relevant art that many additional modifications would be possible without departing from the inventive concepts contained herein. The invention, therefore, is not to be restricted in any manner except in the spirit of the appended claims.

WHAT IS CLAIMED IS:

1. A method for the physical separation or enrichment of cells from a heterogeneous cell population, so as to reduce the total number of phagocytic cells, such as macrophages, monocytes, and/or neutrophils, within the heterogeneous cell population, said method comprising the steps of:

(a) contacting a bone marrow or peripheral blood sample containing said heterogeneous cell population with an effective amount of ferrous particles and incubating the resulting reaction mixture until ferrous particle/cell complexes have formed; and

(b) removing said ferrous particle/cell complexes from the heterogeneous cell population by placing the heterogeneous cell population within a magnetic field whereby an enriched cell population is created.

2. An enriched population of cells prepared according to the method of claim 1.

3. A method of treating malignant diseases comprising-the step of administering to a patient in need thereof an effective concentration to enriched cell population of claim 2.

4. A method of treating hematological diseases comprising the step of administering to a patient in need thereof an effective concentration to enriched cell population of claim 2.

5. The method of claim 1 wherein said enriched cell population is further enriched for hematopoietic $CD34^+$ or myeloid progenitor cells by performing density gradient centrifugation, utilizing Ficoll-Hypaque or Percoll as the density gradient medium, following the initial enrichment with said ferrous particles.

6. An enriched population of cells prepared according to the method of claim 5.

7. A method of treating malignant diseases comprising the step of administering to a patient in need thereof an effective concentration to enriched cell population of claim 6.

8. A method of treating hematological diseases comprising the step of administering to a patient in need thereof an effective concentration to enriched cell population of claim 6.

9. The method of claim 1 wherein the cell population, previously enriched by the ferrous-based magnetic separation methodology, is further enriched for hematopoietic $CD34^+$ or myeloid progenitor cells by:

(a) contacting said enriched cell population cells with paramagnetic iron-containing polystyrene particles conjugated with an appropriate monoclonal antibody which is directed against a desired hematopoietic cell-surface antigen wherein an antibody-conjugated paramagnetic particle/cell complex is formed; and

(b) removing the antibody-conjugated paramagnetic particle/cell complexes by the application of a magnetic field.

10. The method of claim 9 wherein the monoclonal antibody of step (a) is an anti-CD34 monoclonal antibody.

11. An enriched population of cells prepared according to the method of claim 9.

12. A method of treating malignant diseases comprising the step of administering to a patient in need thereof an effective concentration to enriched cell population of claim 11.

13. A method of treating hematological diseases comprising the step of administering to a patient in need thereof an effective concentration to enriched cell population of claim 11.

14. The method of claim 5 wherein said cell population, previously enriched by both the ferrous-based magnetic separation and density gradient centrifugation methodologies, is further enriched for hematopoietic $CD34^+$ or myeloid progenitor cells by:

(a) contacting said enriched cell population cells with paramagnetic iron-containing polystyrene particles conjugated with an appropriate monoclonal antibody which is directed against a desired hematopoietic cell-surface antigen wherein an antibody-conjugated paramagnetic particle/cell complex is formed; and

(b) removing the antibody-conjugated paramagnetic panicle/cell complexes by the application of a magnetic field.

15. The method of claim 14 wherein the monoclonal antibody of step (a) is an anti-CD34 monoclonal antibody.

16. An enriched population of cells prepared according to the method of claim 14.

17. A method of treating malignant diseases comprising the step of administering to a patient in need thereof an effective concentration to enriched cell population of claim 16.

18. A method of treating hematological diseases comprising the step of administering to a patient in need thereof an effective concentration to enriched cell population of claim 16.

19. The method of claim 1 wherein the cell population, previously enriched by the ferrous-based magnetic separation methodology, is further enriched for hematopoietic $CD34^+$ or myeloid progenitor cells by:

(a) contacting said enriched cell population cells with paramagnetic iron-containing polystyrene particles conjugated with an appropriate monoclonal antibody which is directed against a desired hematopoietic cell-surface antigen wherein an antibody-conjugated paramagnetic particle/cell complex is formed; and

(b) removing the antibody-conjugated paramagnetic particle/cell complexes by the application of a magnetic field.

20. The method of claim 19 wherein the monoclonal antibody of step (a) is an anti-CD34 monoclonal antibody.

21. An enriched population of cells prepared according to claim 19.

22. A method of treating malignant diseases comprising the step of administering to a patient in need thereof an effective concentration to enriched cell population of claim 21.

23. A method of treating hematological diseases comprising the step of administering to a patient in need thereof an effective concentration to enriched cell population of claim 21.

24. The method of claim 5 wherein said cell population, previously enriched by both the ferrous-based magnetic separation and density gradient centrifugation methodologies, is further enriched for hematopoietic $CD34^+$ or myeloid progenitor cells by:

(a) contacting said enriched cell population cells with an appropriate monoclonal antibody, which is directed against a desired hematopoietic cell-surface antigen, conjugated to a solid support matrix wherein an antibody-conjugated solid support matrix/cell complex is formed; and

(b) removing the enriched cells from the anti body-conjugated solid support matrix/cell complexes by mechanical, chemical, or enzymatic means.

25. The method of claim 24 wherein the monoclonal antibody of step (a) is an anti-$CD34^+$ monoclonal antibody.

26. An enriched population of cells prepared according to claim 24.

27. A method of treating malignant diseases comprising the step of administering to a patient in need thereof an effective concentration to enriched cell population of claim 26.

28. A method of treating hematological diseases comprising the step of administering to a patient in need thereof an effective concentration to enriched cell population of claim 26.

29. The method of claim 1 wherein the cell population, previously enriched by the ferrous-based magnetic separation methodology, is purged of any contaminating, malignant tumor cells by:

(a) contacting said enriched cell population with paramagnetic iron-containing polystyrene particles conjugated with an appropriate monoclonal antibody which is directed against a desired cell-surface antigen

present on the malignant cell wherein an antibody-conjugated paramagnetic particle/malignant cell complex is formed; and

(b) removing the antibody-conjugated paramagnetic particle/malignant cell complexes by the application of a magnetic field

30. The method of claim 29 wherein the monoclonal antibody of step (a) is an anti-neuroblastoma monoclonal antibody.

31. An enriched population of cells prepared according to claim 29.

32. A method of treating malignant diseases comprising the step of administering to a patient in need thereof an effective concentration to enriched cell population of claim 31.

33. A method of treating hematological diseases comprising the step of administering to a patient in need thereof an effective concentration to enriched cell population of claim 31.

34. The method of claim 5 wherein said cell population. previously enriched by both the ferrous-based magnetic separation and density gradient centrifugation methodologies, is purged of any contaminating, malignant tumor cells by:

(a) contacting said enriched cell population with paramagnetic iron-containing polystyrene particles conjugated with an appropriate monoclonal antibody which is directed against a desired cell-surface antigen present on the malignant cell wherein an antibody-conjugated paramagnetic particle/malignant cell complex is formed; and

(b) removing the antibody-conjugated paramagnetic particle/malignant cell complexes by the application of a magnetic field.

35. The method of claim 34 wherein the monoclonal antibody of step (a) is an antineuroblastoma monoclonal antibody.

36. An enriched population of cells prepared according to claim 34.

37. A method of treating malipant diseases comprising the step of administering to a patient in need thereof an effective concentration to enriched cell population of claim 36.

38. A method of treating hematological diseases comprising the step of administering to a patient in need thereof an effective concentration to enriched cell population of claim 36.

39. The method of claim 5 wherein said cell population, previously enriched by both the ferrous-based magnetic separation and density gradient centrifugation methodologies, is purged of any contaminating, malignant tumor cells by:

(a) contacting said enriched cell population cells with an appropriate monoclonal antibody which is directed against a desired cell-surface antigen of the malignant cell, conjugated to a solid support matrix wherein an antibody-conjugated solid support matrix/malignant cell complex is formed; and

(b) removing the tumor-free cells from the antibody-conjugated solid support matrix/malignant cell complexes by mechanical, chemical, or enzymatic means.

40. The method of claim 39 wherein the monoclonal antibody of step (a) is an anti-neuroblastoma monoclonal antibody.

41. An enriched population of cells prepared according to claim 39.

42. A method of treating malignant diseases comprising the step of administering to a patient in need thereof an effective concentration to enriched cell population of claim 41.

43. A method of treating hematological diseases comprising the step of administering to a patient in need thereof an effective concentration to enriched cell population of claim 41.

44. The method of claim 14 wherein the enriched hematopoletic cell population is purged of any contaminating, malignant tumor cells by:

(a) contacting said enriched hematopoietic cell population cells with an appropriate monoclonal antibody, which is directed against a desired cell-surface antigen of the malignant cell, conjugated to a solid support matrix wherein an antibody-conjugated solid support matrix/malignant cell complex is formed; and

(b) removing the tumor-free cells from the antibody-conjugated solid support matrix/ malignant cell complexes by mechanical, chemical, or enzymatic means.

45. The method of claim 44 wherein the monoclonal antibody of step (a) is an anti-neuroblastoma monoclonal antibody.

46. An enriched population of cells prepared according to claim 44.

47. A method of treating malignant diseases comprising the step of administering to a patient in need thereof an effective concentration to enriched cell population of claim 46.

48. A method of treating hematological diseases comprising the step of administering to a patient in need thereof an effective concentration to enriched cell population of claim 46.

49. The method of claim 24 wherein the enriched hematopoietic cell population is purged of any contaminating, malignant tumor cells by:

(a) contacting said enriched hematopoietic cell population cells with an appropriate monoclonal antibody, which is directed against a desired cell-surface antigen of the malignant cell, conjugated to a solid support matrix wherein an antibody-conjugated solid support matrix/malignant cell complex is formed; and

(b) removing the tumor-free cells from the antibody-conjugated solid support matrix/malignant cell complexes by mechanical, chemical, or enzymatic means.

50. The method of claim 49 wherein the monoclonal antibody of step (a) is an anti-neuroblastoma monoclonal antibody.

51. An enriched population of cells prepared according to claim 49.

52. A method of treating malignant diseases comprising the step of administering to a patient in need thereof an effective concentration to enriched cell population of claim 51.

53. A method of treating hematological diseases comprising the step of administering in an effective concentration to enriched cell population of claim 49.

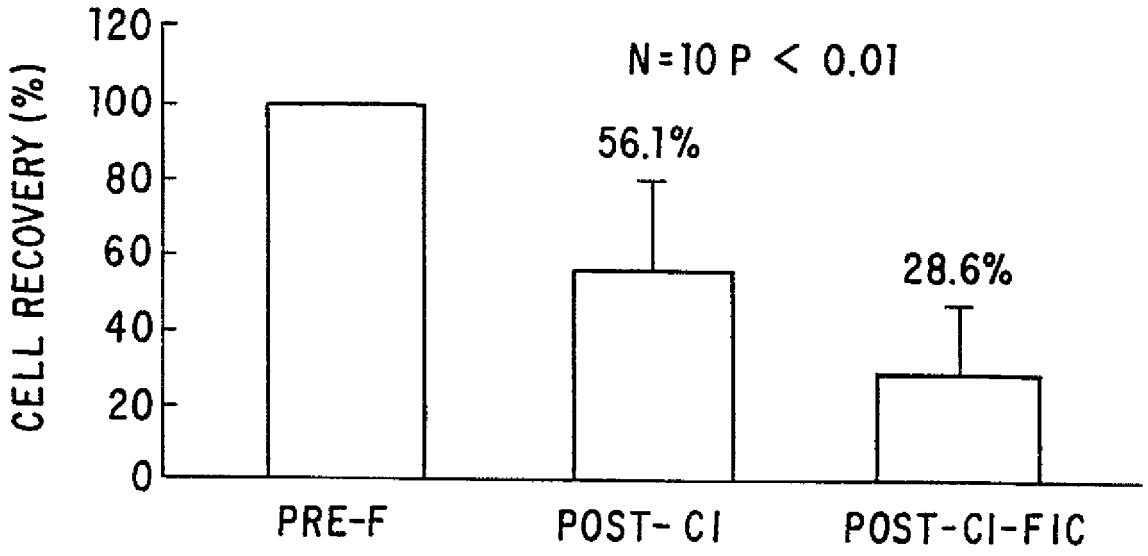
CELL RECOVERY (%)
120
100
80
60
40
20
0
N=10 P < 0.01
56.1%
28.6%
PRE-F
POST- CI
POST-CI-FIC

FIG. 1A

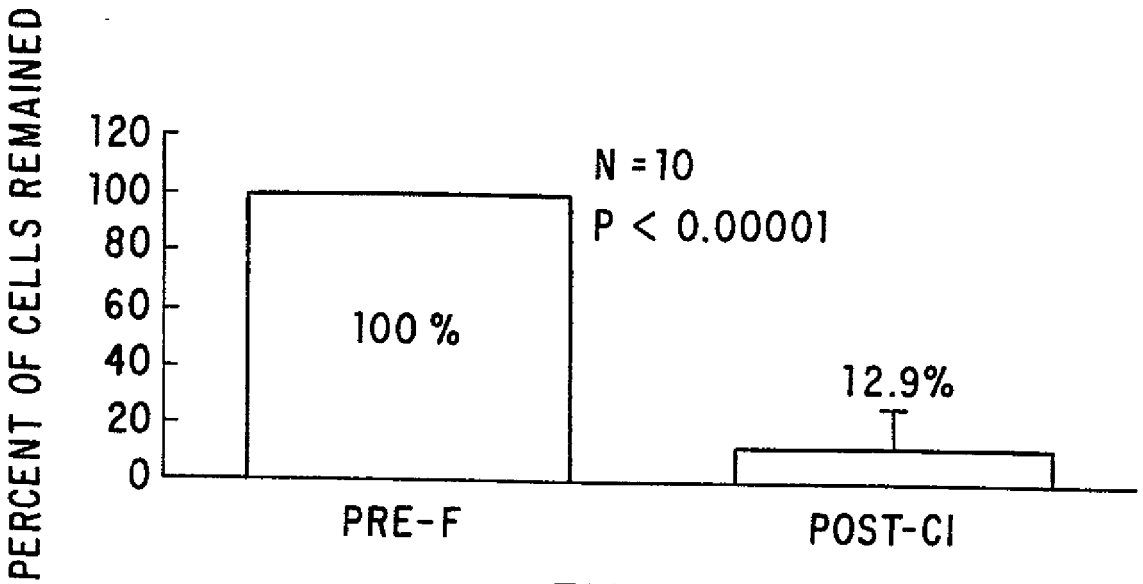
PERCENT OF CELLS REMAINED
120
100
80
60
40
20
0
N = 10
P < 0.00001
100 %
12.9%
PRE-F
POST-CI

FIG. 1B

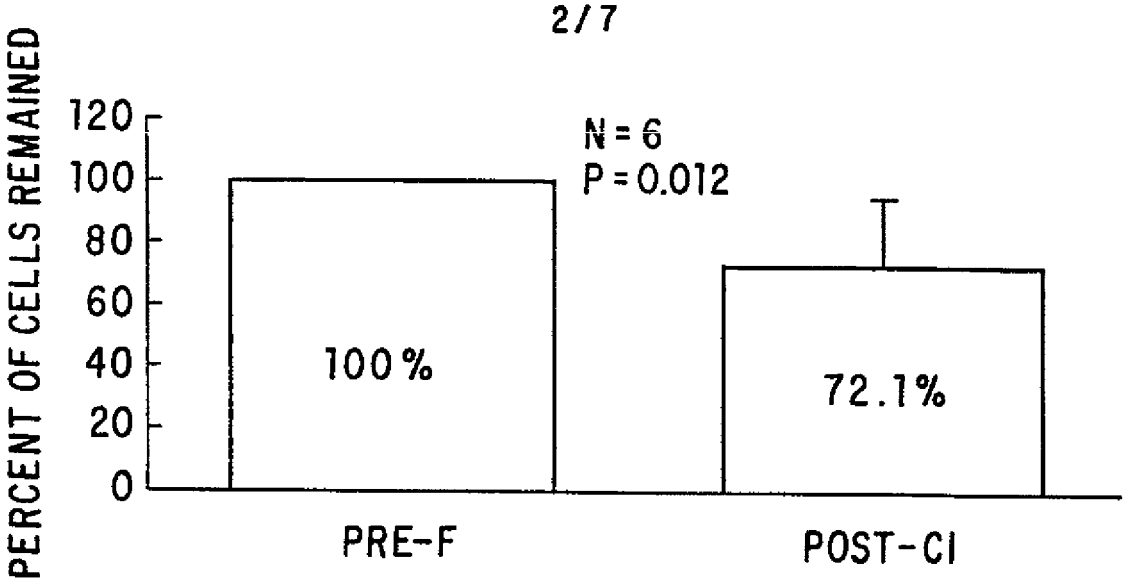
PERCENT OF CELLS REMAINED
120
100
80
60
40
20
0
N = 6
P = 0.012
100%
72.1%
PRE-F
POST-CI

FIG. 1C

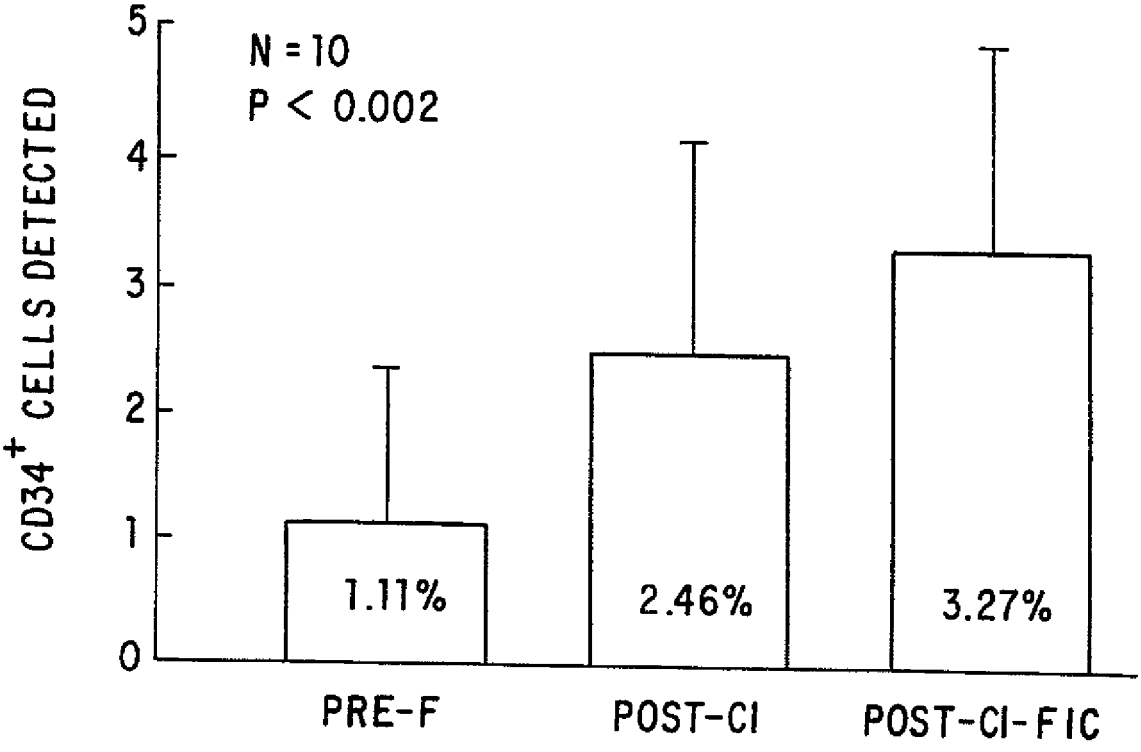
CD34+ CELLS DETECTED
5
4
3
2
1
0
N = 10
P < 0.002
1.11%
2.46%
3.27%
PRE-F
POST-CI
POST-CI-FIC

FIG. 2A

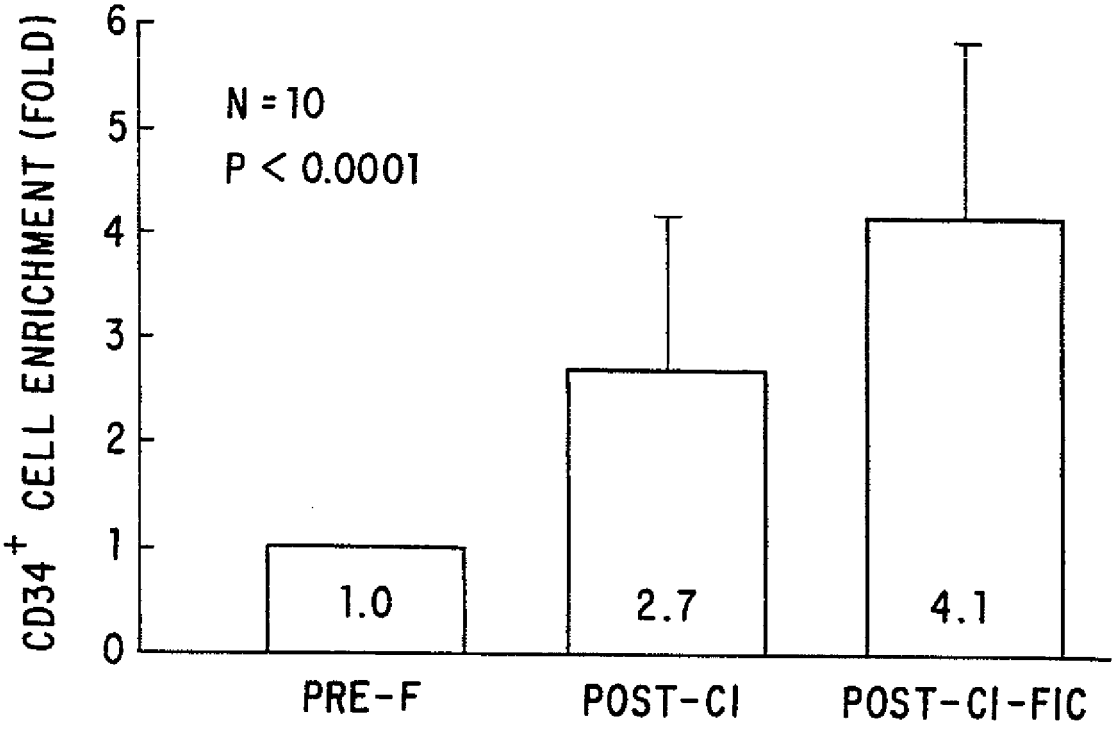
CD34+ CELL ENRICHMENT (FOLD)
6
5
4
3
2
1
0
N = 10
P < 0.0001
1.0
2.7
4.1
PRE-F
POST-CI
POST-CI-FIC

FIG. 2B

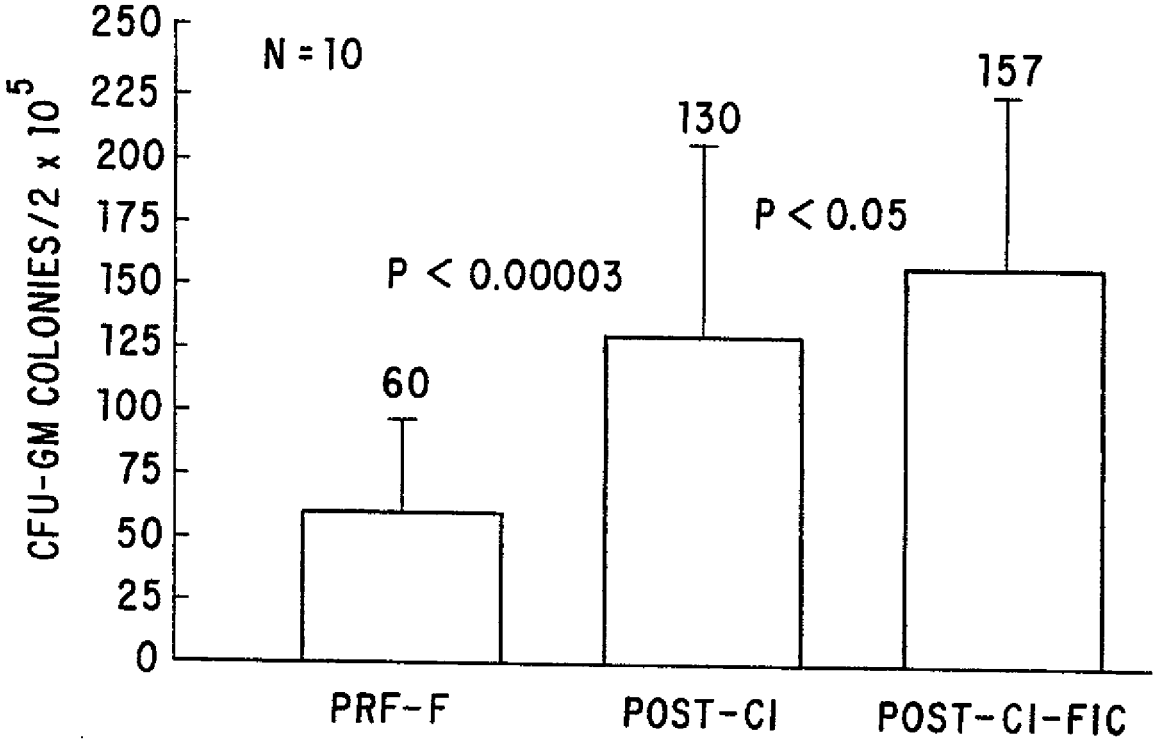
CFU-GM COLONIES/2 x 10^5
250
225
200
175
150
125
100
75
50
25
0
N = 10
P < 0.00003
P < 0.05
60
130
157
PRF-F
POST-CI
POST-CI-FIC

FIG. 3A

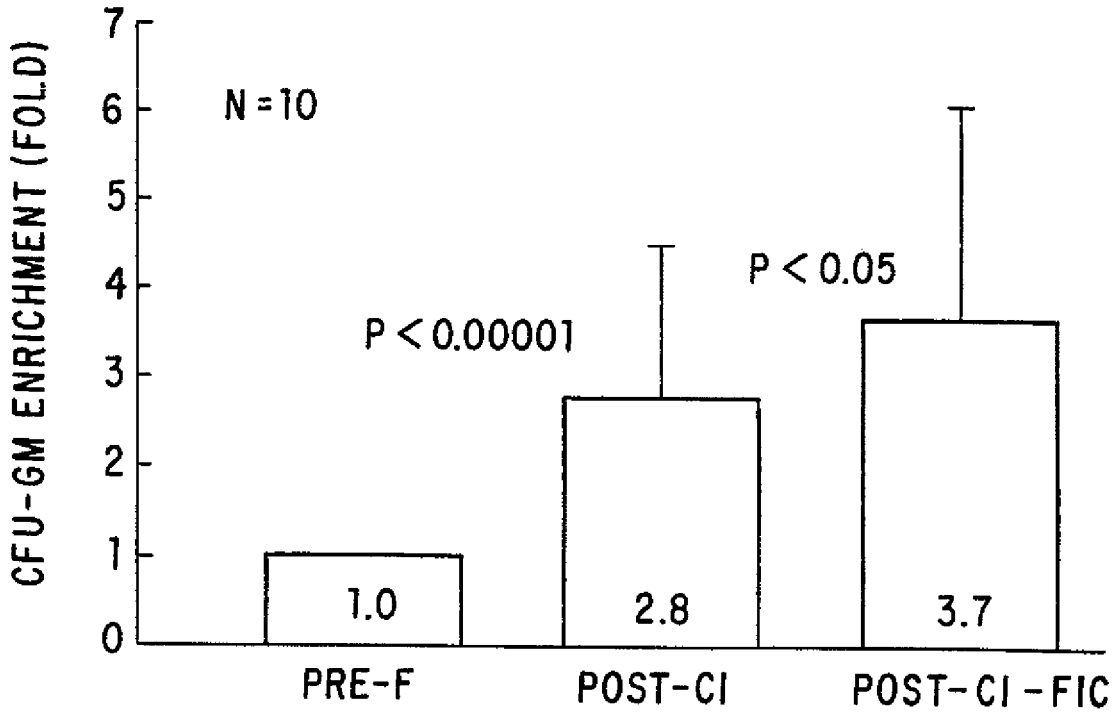
CFU-GM ENRICHMENT (FOLD)
7
6
5
4
3
2
1
0
N = 10
P < 0.00001
P < 0.05
1.0
2.8
3.7
PRE-F
POST-CI
POST-CI-FIC

FIG. 3B

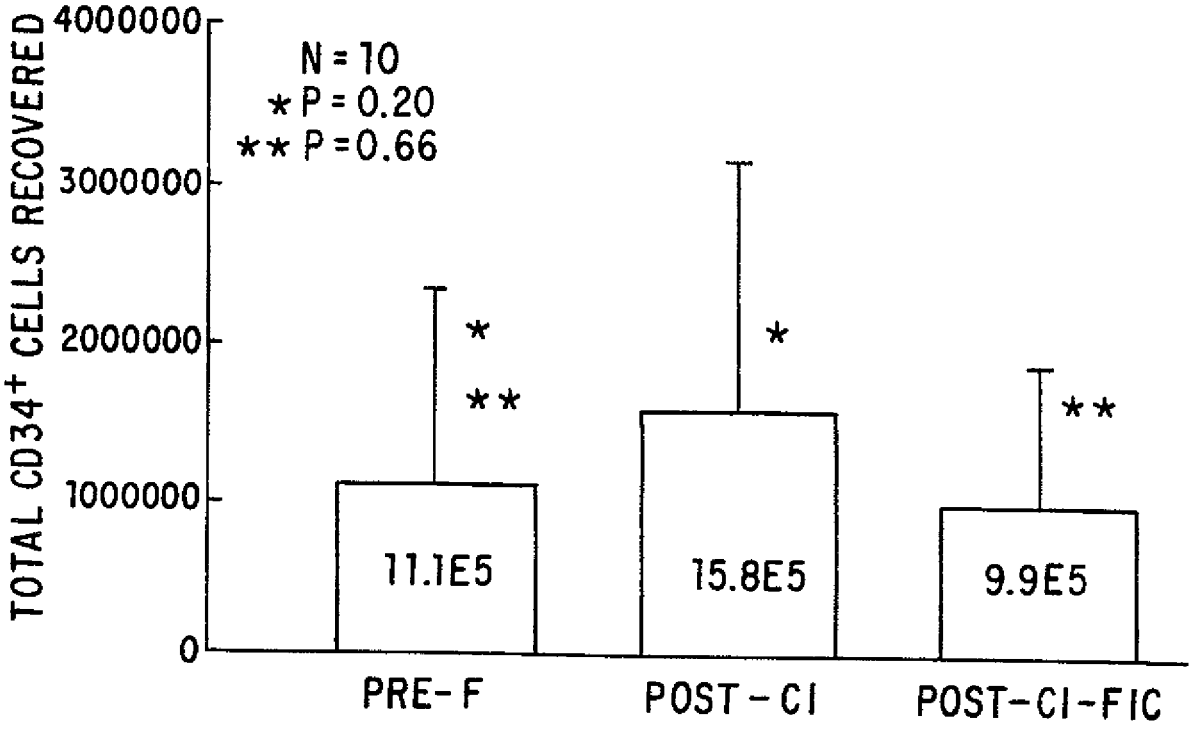
TOTAL CD34+ CELLS RECOVERED
4000000
3000000
2000000
1000000
0
N = 10
* P = 0.20
** P = 0.66
*
**
*
**
11.1E5
15.8E5
9.9E5
PRE-F
POST-CI
POST-CI-FIC

FIG. 4A

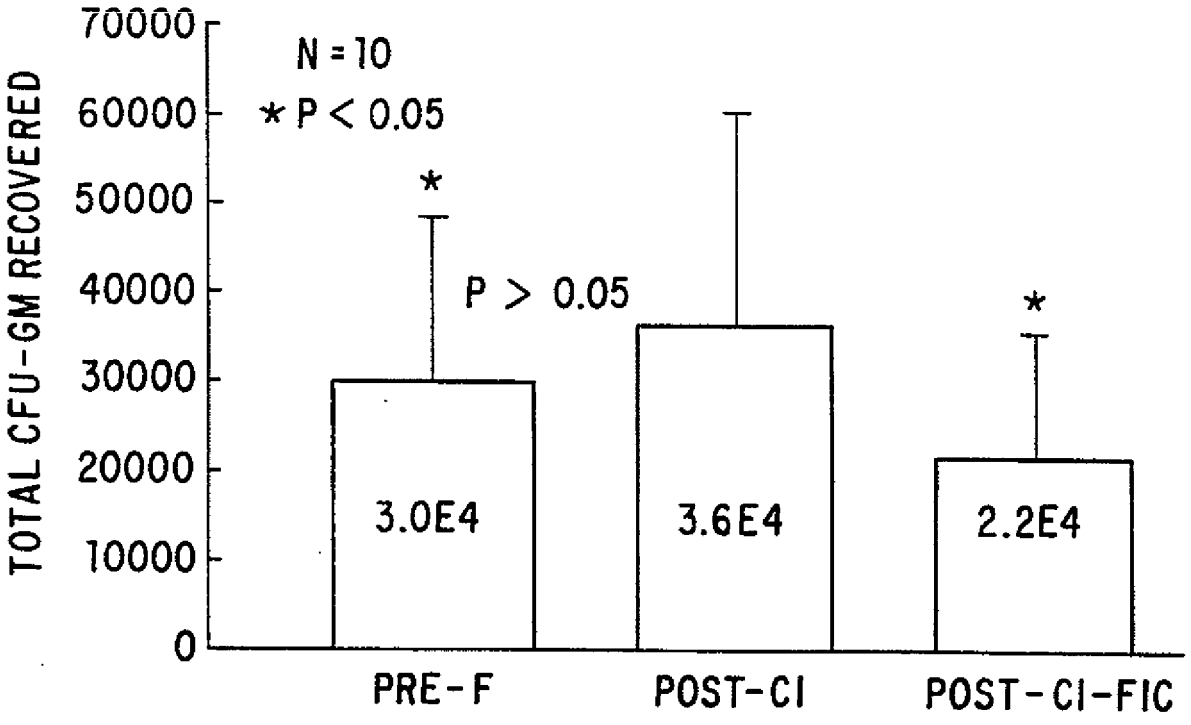
TOTAL CFU-GM RECOVERED
70000
60000
50000
40000
30000
20000
10000
0
N = 10
* P < 0.05
*
P > 0.05
*
3.0E4
3.6E4
2.2E4
PRE-F
POST-CI
POST-CI-FIC

FIG. 4B

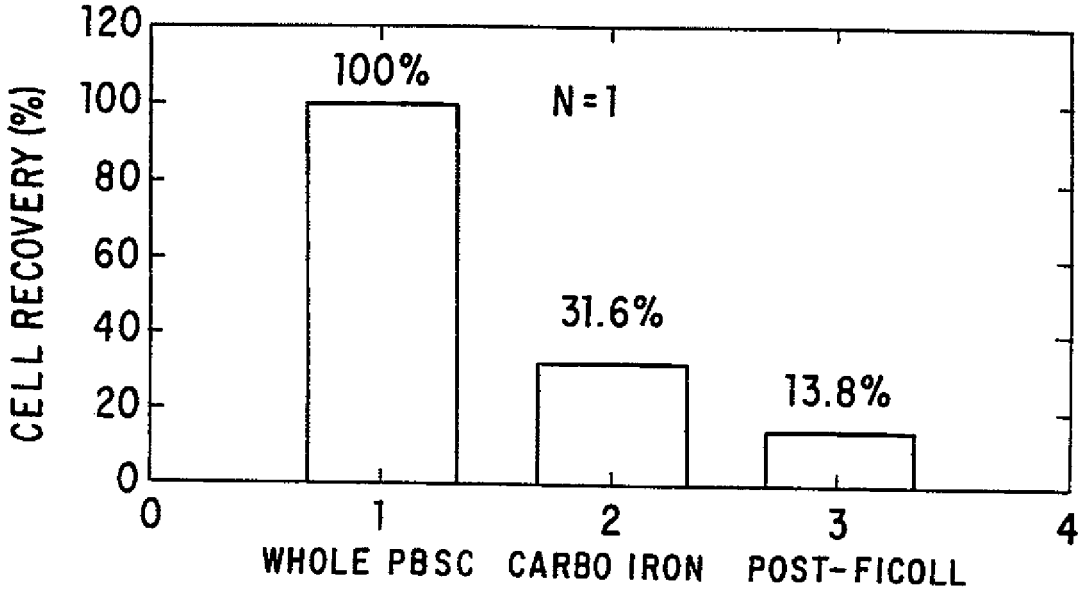
CELL RECOVERY (%)
120
100
80
60
40
20
0
100%
N = 1
31.6%
13.8%
0
1
2
3
4
WHOLE PBSC
CARBO IRON
POST-FICOLL

FIG. 5A

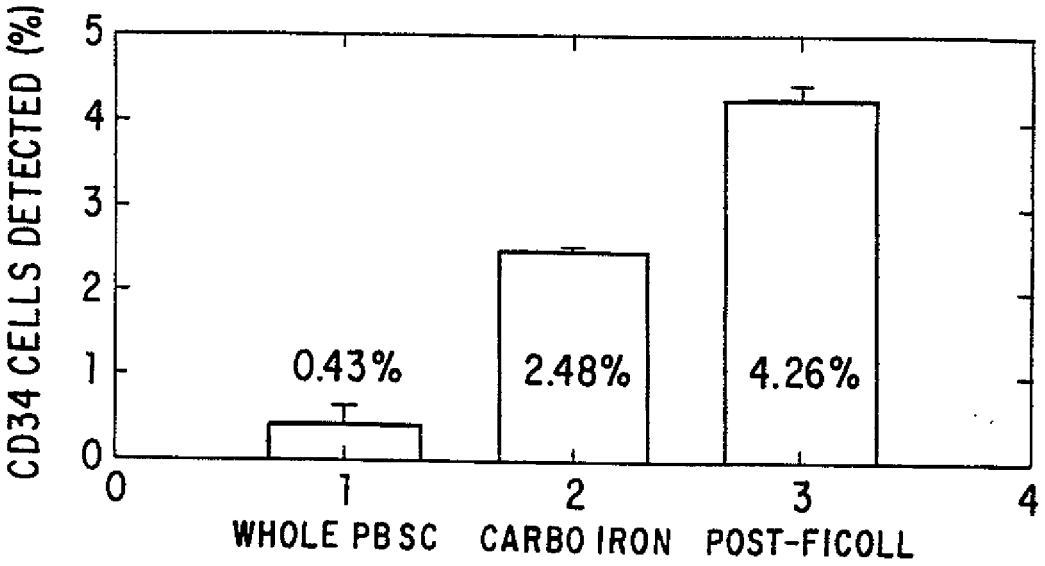
CD34 CELLS DETECTED (%)
5
4
3
2
1
0
0.43%
2.48%
4.26%
0
1
2
3
4
WHOLE PB SC
CARBO IRON
POST-FICOLL

FIG. 5B

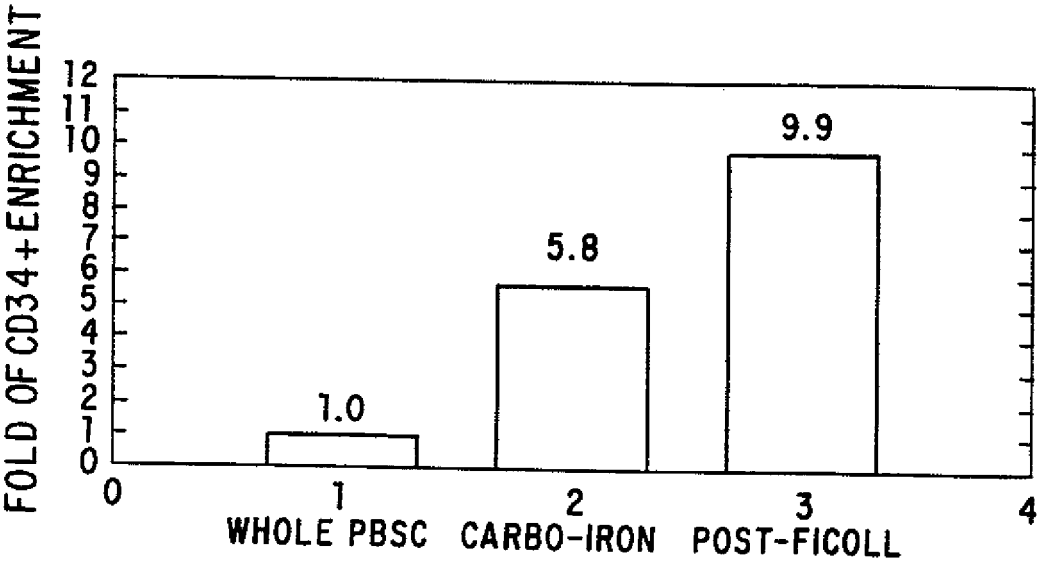
FOLD OF CD34+ENRICHMENT
12
11
10
9
8
7
6
5
4
3
2
1
0
1.0
5.8
9.9
0
1
2
3
4
WHOLE PBSC
CARBO-IRON
POST-FICOLL

FIG. 5C

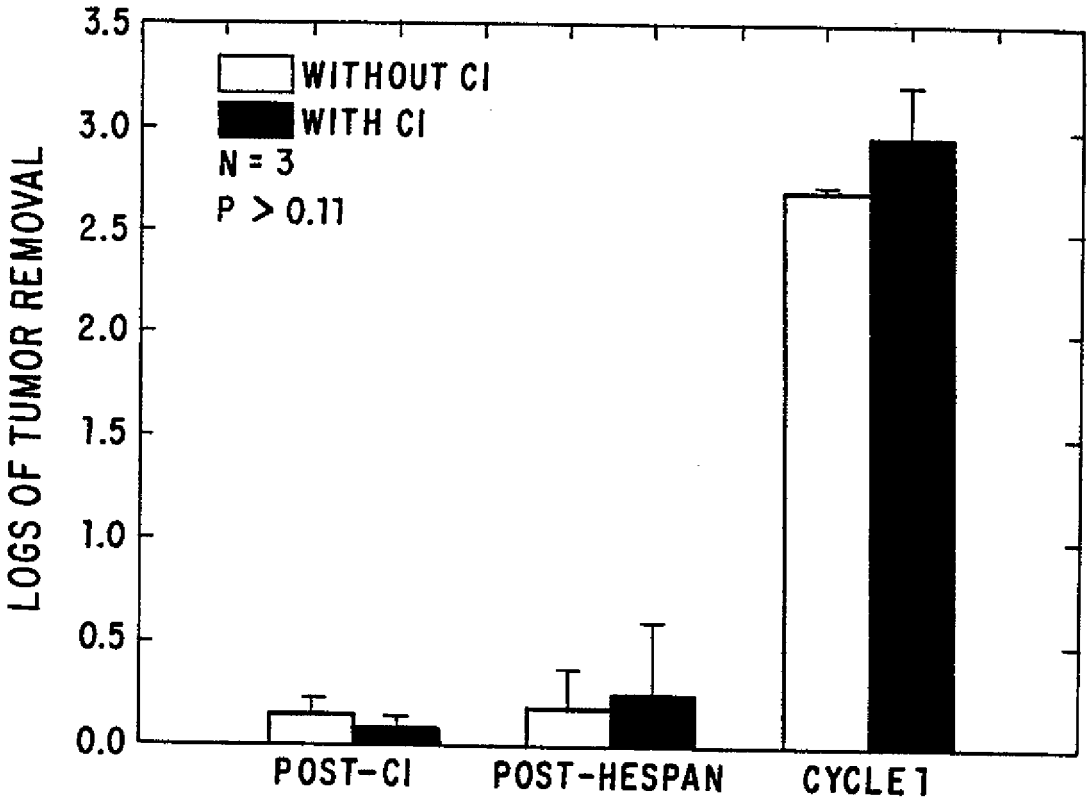
LOGS OF TUMOR REMOVAL
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
WITHOUT CI
WITH CI
N = 3
P > 0.11
POST-CI
POST-HESPAN
CYCLE 1

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/07440

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12M 3/00, 3/02; C12N 5/06, 5/08; A61K 35/12, 35/26, 35/28
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/173.9, 180, 240.2, 284, 324, 378, 379, 395, 402, 403; 424/93.7, 93.71

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WONG et al. Depletion of Macrophages from Heterogenous Cell Populations by Use of Carbonyl Iron. Methods in Enzymology. 1994. Vol. 108. pages 307-313, see entire document.	1, 2 --- 3-53
Y	US 3,970,518 A (GIAEVER) 20 July 1976, see enitre document.	9-23, 39-48
Y	MILTENYI et al. High Gradient Magnetic Cell Separation With MACS. Cytometry. 1990. Vol. 11. pages 231-238, see entire document.	9-23, 39-48
Y	REYNOLDS et al. An Immunomagnetic Flow System for Selective Depletion of Cell Populations From Marrow. Transplantation Proc. 1985. Vol. 17. No. 1. pages 434-436, see entire document.	3-4, 7-8, 12-13, 17-18, 22-23, 27-53

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 08 JULY 1998	Date of mailing of the international search report 13 AUG 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer NANCY A. JOHNSON Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/07440

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,081,030 A (CIVIN) 14 January 1992, see abstract and col. 2, lines 10-14, col 11-12.	3-4, 7-28, 44-53
Y	US 5,409,813 A (SCHWARTZ) 25 April 1995, see abstract, claims 1-25 and col. 3-4.	9-53
Y	US 5,468,612 A (COHEN et al) 21 November 1995, see col. 1, 4, 16-17.	3-4, 9-53

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/07440

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/173.9, 180, 240.2, 284, 324, 378, 379, 395, 402, 403; 424/93.7, 93.71

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, BIOSIS, EMBASE, WPIDS

leukocytes, macrophages, cell separation, magnetic, anti-CD34, anti-neuroblastoma